Proc. Natl. Acad. Sci. USA
Vol. 78, No. 9, pp. 5886–5889, September 1981
Neurobiology

Thiamine as an integral component of brain synaptosomal membranes

(brain subcellular fractions)

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Communicated by Alfred Gilman, May 14, 1981

ABSTRACT **Synaptic plasma membranes were prepared from rat cerebral cortex to determine if thiamine was localized in the membranes. The synaptosomes, prepared by discontinuous sucrose gradient centrifugation, were subjected to osmotic shock at pH 9.5 for 10–15 min and subfractionated on a discontinuous sucrose gradient. The two membrane fractions that were obtained were free of contamination by mitochondrial membrane and soluble fractions. In order to ensure specificity, thiamine was assayed fluorometrically before and after the addition of thiaminase I (thiamin:base 2-methyl-4-aminopyrimidine-5-methenyltransferase, EC 2.5.1.2). The thiamine content of the two membrane fractions was 9 and 10 pmol/mg protein.**

That thiamine may play a role in nerve conduction is suggested by the observations that antimetabolites of the vitamin affect axonal conduction (1–4), thiamine is released from nerve preparations in response to electrical stimulation or the application of neuroactive drugs such as tetrodotoxin (5, 6), and thiamine restores excitability to ultraviolet irradiated nerve fibers (7) or to a voltage-clamped node of Ranvier preparation that has run down with time (8).

If the vitamin is associated with electrical excitability, then one would expect to find it localized in the plasma membrane. Although fluorescence histochemical experimentation has suggested this localization (9, 10), this technique does not permit a precise determination. For this reason, this project was undertaken. Three enzyme systems in the brain use thiamine pyrophosphate (ThPP) as coenzyme. These are transketolase (sedoheptulose-7-phosphate: D-glyceraldehyde-3-phosphate glycolaldehydetransferase, EC 2.2.1.1), pyruvate dehydrogenase [pyruvate:lipoamide oxidoreductase (decarboxylating and acceptor-acetylating), EC 1.2.4.1]; and α-ketoglutarate dehydrogenase [2-oxoglutarate:lipoamide oxidoreductase (decarboxylating and acceptor-succinylating), EC 1.2.4.2]. Because transketolase is a soluble enzyme and ThPP dissociates readily from pyruvate dehydrogenase, the remaining concern with respect to a possible contaminant of the plasma membrane fraction is α-ketoglutarate dehydrogenase. Accordingly, this enzyme activity was assayed during the purification procedure.

MATERIALS AND METHODS

Materials. ThPP was obtained from Calbiochem-Behring. ATP (vanadate-free), NAD^+, CoA, and cytochrome *c* (type III) were obtained from Sigma. [^{35}S]Thiamine was obtained from Amersham. Thiamine triphosphate (ThTP) was a gift from Sankyo (Tokyo). All other reagents were of analytical grade.

Methods. Ouabain-sensitive Na^+,K^+-ATPase (ATP phosphohydrolase, EC 3.6.1.3) was assayed at 37°C by determining the difference in phosphate release in the absence and presence of 0.2 mM ouabain (11). α-Ketoglutarate dehydrogenase was assayed as described by Holowach *et al.* (12). Cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) was assayed by the method of Wharton and Tzagoloff (13); the value 29.5 was used as the millimolar coefficient of reduced cytochrome *c* at 550 nm. Protein was determined by the method of Lowry *et al.* (14) with bovine serum albumin as standard. All enzyme reactions were linear over the range studied.

Thiamine Assay. Thiamine was extracted from each preparation (1–3 mg of protein per ml) with trichloroacetic acid at 5% (wt/vol; final concentration). After removal of the trichloroacetic acid by four extractions with ether, thiamine was determined by the thiochrome reaction with alkaline cyanogen bromide as the oxidizing reagent (15). In each tissue blank, the oxidizing reagent was added after the alkaline solution to destroy thiamine. The fluorescence was measured at 365 nm excitation and 440 nm emission wavelengths with ThPP (5–200 pmol) as standard. In order to ensure that authentic thiamine was being assayed, in some experiments thiaminase I (thiamin:base 2-methyl-4-aminopyrimidine-5-methenyltransferase, EC 2.5.1.2; 1823 milliunits/mg of protein) prepared by the method of Witliff and Airth (16) was used as follows. In a final volume of 3 ml, 0.03 M sodium phosphate buffer (pH 5.8), sample, and 0.4–0.6 mg of enzyme were incubated for 60 min at 37°C. The solution was then assayed for remaining thiamine.

Electron Microscopy. R. A. Schulz kindly provided the electron microscopic examination of the subfractions. The procedure was as described (17).

Preparation of Synaptic Plasma Membranes. Sprague–Dawley rats weighing 80–100 g were used. The cerebral cortices from four to six rats were homogenized in 9 vol of 0.32 M sucrose/0.1 mM EDTA/1 mM sodium phosphate, pH 7.4, by a motor-driven Teflon/glass homogenizer. The crude mitochondrial pellet was obtained from the homogenate by the procedure of Gurd *et al.* (18). The pellet was washed twice and resuspended in the homogenizing solution (4 ml/g of tissue). The suspension was layered over a discontinuous sucrose gradient consisting of successive 8-ml layers of 0.8 M, 1.0 M, and 1.2 M sucrose containing 0.1 mM EDTA and 1 mM sodium phosphate (pH 7.4) and centrifuged at 63,000 × *g* for 2 hr in a Beckman SW 25.1 rotor. Both the synaptosomal fraction at the 1.0 M–1.2 M sucrose interface and the lower layer were removed, diluted with 3 vol of 0.1 mM EDTA/1 mM sodium phosphate, pH 7.4, and centrifuged at 20,000 × *g* for 20 min.

The pellet was resuspended in a small volume of the homogenizing solution and diluted with 15 vol of 0.1 mM EDTA/

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Abbreviations: ThPP, thiamine pyrophosphate; ThTP, thiamine triphosphate.

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1 mM sodium phosphate, pH 7.4. The pH of the suspension was then adjusted to pH 9.5 by the addition of 0.05 M NaOH. The suspension was allowed to stand for 10–15 min at 0°C and homogenized for 1 min (three strokes). The pH was then brought back to neutrality by the addition of 0.05 M HCl and the suspension was centrifuged at 78,000 × g for 30 min in a Beckman type 30 rotor. The resulting pellet was resuspended in the homogenizing solution and layered over a discontinuous sucrose gradient consisting of successive 6-ml layers of 0.8 M, 1.0 M, 1.1 M, and 1.2 M sucrose containing 0.1 mM EDTA and 1 mM sodium phosphate (pH 7.4). After centrifugation at 63,000 × g for 1.5 hr, each fraction was removed, diluted with 0.1 mM EDTA/1 mM sodium phosphate, pH 7.4, and pelleted at 78,000 × g for 30 min. The pellets were resuspended in 0.32 M sucrose for assay. All procedures were carried out at 4°C.

RESULTS

Table 1 shows the distribution of protein, some enzyme activities, and the thiamine content in the fractions obtained from rat cerebral cortex. The specific activity of Na^+,K^+-ATPase activity, a marker for synaptic plasma membranes (18–23), was highest in fractions 1 and 2, and 23% and 29% of the total activity in lysed synaptosomes were recovered in these fractions, respectively. Cytochrome *c* oxidase activity was used as a marker for mitochondria (19–21, 23). We also assayed the activity of α-ketoglutarate dehydrogenase, a mitochondrial ThPP-dependent enzyme (12), to determine if the coenzyme might contribute to the determination of thiamine in the membranes. α-Ketoglutarate dehydrogenase activity was not detectable in fractions 1 and 2 but appeared in fractions 3–5. The same tendency was observed in the distribution of cytochrome *c* oxidase activity which was not detectable in fraction 1 and was low in fraction 2 (1.6% of that in fraction 5). Electron microscopic examination of fractions 1 and 2 (Fig. 1) showed that these fractions were membranous, although fraction 1 was slightly contaminated by myelin. The yield of synaptic plasma membrane protein recovered in fractions 1 and 2 was 1.4 and 1.5 mg/g (wet weight) of cortex, respectively. To check for contamination with the soluble fraction, we homogenized the cerebral cortex with a homogenizing solution containing [^{35}S]thiamine (10^6 cpm) and fractionated it; radioactivity was not detectable in fractions 1 and 2 (data not shown).

The content of thiamine was highest in fraction 5, and the distribution was similar to that of mitochondrial enzyme activities (Table 1). However, it was also present in fractions 1 and 2. To confirm that the thiamine assayed here was authentic, we examined the effect of thiaminase I, which catalyzes specifically the decomposition of thiamine (16), on the trichloroacetic acid extracts (Table 2). After the enzyme treatment, the fluorescence in fractions 1, 2, and 5 decreased to 22%, 31%, and 32%, respectively. Authentic thiamine was completely decomposed under these conditions. Thus, some material in the extract yields the same fluorophore but is not the vitamin. When this correction was made, the amount of authentic thiamine found in the two membrane fractions amounted to 9 and 10 pmol/mg of protein.

DISCUSSION

Synaptic plasma membranes previously have been prepared after osmotic shock of synaptosomes prepared on discontinuous gradients of sucrose (24, 25), Ficoll (18, 19, 23), sodium diatrizoate (22), or a combination of sucrose and $CsCl_2$ gradients (20). Osmotic shock has been usually carried out at pH 8.0–8.5 because Cotman and Matthews (19) reported that an alkaline condition was effective in separating membranes from mitochondria. However, with this procedure, membranes were still contaminated by mitochondria by approximately 7%. Recently, Mena *et al.* (23) reported an improved method of preparing synaptic membranes almost devoid of 2′,3′-cyclic nucleotide 3′-phosphohydrolase activity. Although this preparation provides membranes that are less contaminated by mitochondria, the method is lengthy and the yield is low.

Davis and Bloom (26) and Cotman and Taylor (27), as an approach to reducing mitochondrial contamination, used *p*-indonitroneotetrazolium violet which interacts with succinate dehydrogenase in mitochondria to increase the density. Our preliminary experiments with this method showed that this treatment inactivated Na^+,K^+-ATPase and α-ketoglutarate dehydrogenase and, in addition, it was difficult to wash out the pigment. We found that the separation of membranes and mitochondria was more effective at pH 9.5 than at pH 8.5 and was effective with a shorter osmotic shock. Furthermore, the enzyme activity assayed here was not lost during the preparation.

Na^+,K^+-ATPase activity was recovered in fraction 1 (0.32–0.8 M) and 2 (0.8–1.0 M); the highest specific activity, found in fraction 2, was 6.5 times that of the homogenate (0.30 ± 0.02 μmol/mg of protein per min in five experiments). This enrichment is in agreement with the theoretical value (5- to 10-fold) estimated by Cotman and Matthews (19). It is unlikely that α-ketoglutarate dehydrogenase was inactivated in our procedure because the recovery of enzyme activity was almost complete and, in addition, exogenously added ThPP did not affect the activity.

We cannot specify the origin of the two purified plasmolemma fractions. Conceivably, one fraction may be glial membrane and the other, neuronal plasma membrane, but no un-

Table 1. Enzymatic properties and thiamine content of synaptosomal subfractions

	Protein (n = 5), %	Na^+,K^+-ATPase (n = 5)		α-Ketoglutarate dehydrogenase (n = 5)		Cytochrome *c* oxidase (n = 3)	Thiamine (n = 9), pmol/mg protein
		SA	Total, %	SA	Total, %	SA	
Lysed synaptosomes	100	0.95 ± 0.04	100	70.5 ± 10.3	100	—	—
Fractions							
1 (0.32–0.8 M)	13.2 ± 0.3	1.66 ± 0.14	23.0 ± 2.0	0	0	0	12 ± 2 (9.4)*
2 (0.8–1.0 M)	14.2 ± 0.9	1.96 ± 0.31	29.4 ± 4.0	0	0	0.02 ± 0.00	14 ± 2 (9.7)*
3 (1.0–1.1 M)	9.9 ± 0.2	1.17 ± 0.05	12.1 ± 0.3	25.0 ± 3.1	4.1 ± 0.4	—	—
4 (1.1–1.2 M)	16.0 ± 1.6	0.54 ± 0.06	11.3 ± 2.5	79.6 ± 6.4	23.9 ± 3.3	—	—
5 (pellet)	31.9 ± 1.5	0.15 ± 0.01	5.0 ± 0.6	108.9 ± 11.8	52.6 ± 3.5	1.29 ± 0.05	179 ± 32 (121.7)*

Each value is the mean ± SEM. Specific activities (SA) of enzymes are shown as μmol/mg of protein per min.
* In parentheses, authentic thiamine as determined by thiaminase sensitivity (see Table 2).

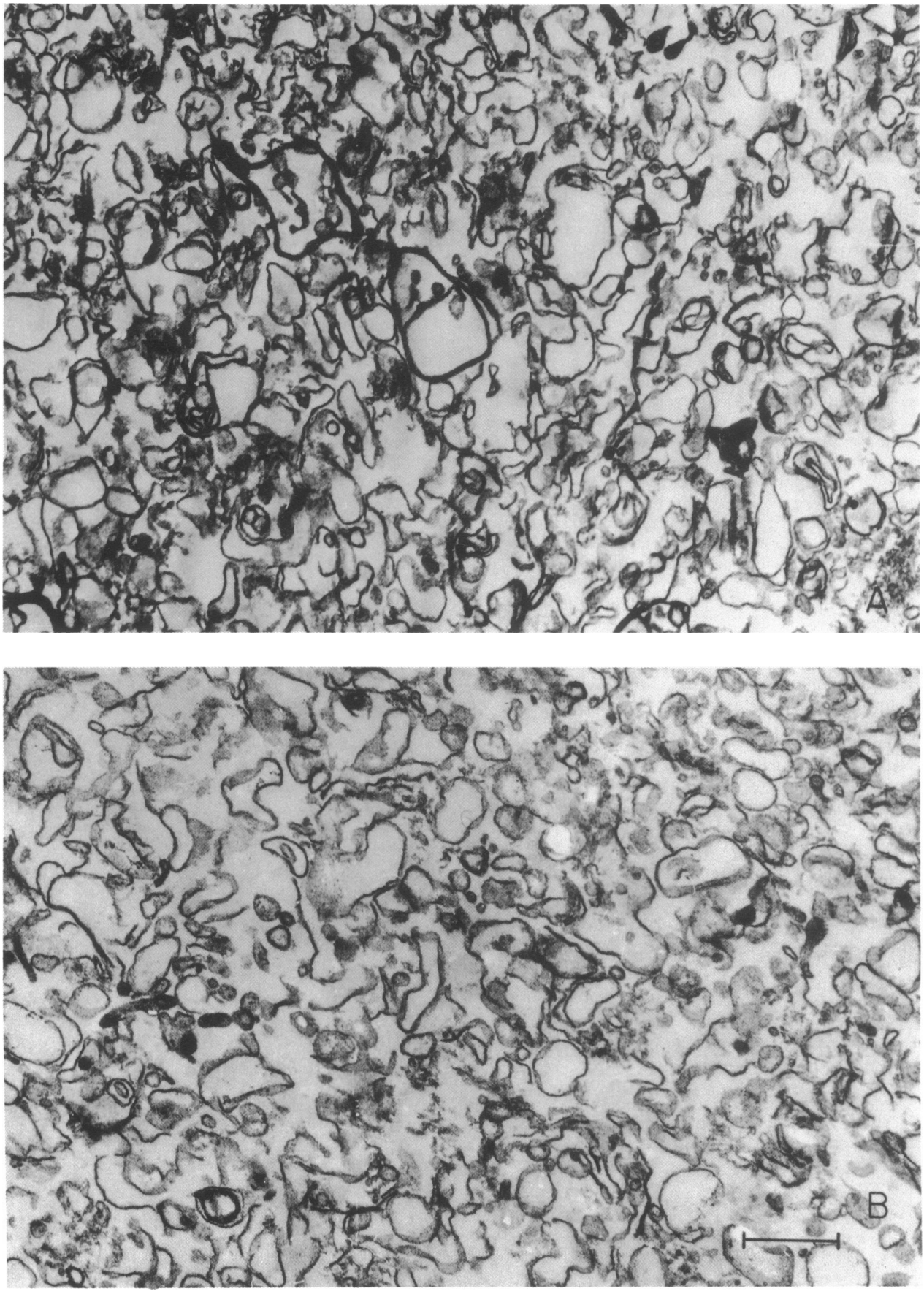

FIG. 1. Electron micrographs of fraction 1 (*A*) and fraction 2 (*B*). (Bar represents 0.5 μm; ×34,000.)

Table 2. Effect of thiaminase I treatment on thiamine content in trichloroacetic acid extracts

	Fraction 1	Fraction 2	Fraction 5	Thiamine
Untreated	100	100	100	100
Thiaminase I	22 ± 4	31 ± 5	32 ± 3	4 ± 1

Extracts from each fraction (corresponding to 0.3–1.3 mg of protein) and authentic thiamine (100 pmol) were incubated with thiaminase I; then, remaining thiamine was assayed. Results, shown as % of control (untreated), represent the mean ± SEM of three experiments.

equivocal markers are available to specify the possibility of the former. Barnola *et al.* (28) prepared membrane fractions from lobster walking nerves and found two bands that were ascribed to schwannlemma and axolemma, but no convincing evidence was presented to support this assignment.

The presence of thiamine in the membrane fractions is documented by the relatively specific alkaline cyanogen bromide oxidation which converts the vitamin to its fluorophore and by the added specificity of incubating the extract with thiaminase I before the addition of the oxidant. It should be noted that the fluorescent procedure measures total thiamine and does not distinguish the free base from its phosphate esters. In separate experiments with a new separation procedure using SP-Sephadex column chromatography, we found that, of the total thiamine in the membrane fractions, approximately 72% is in the form of ThPP, 17% is ThTP, and the remaining 11% is in the monophosphate form (29).

Although considerable evidence suggests a noncoenzyme role of the vitamin, particularly in Leigh disease (see ref. 30 for review), with the current demonstration that thiamine is an integral constituent of membranes, it is pertinent to raise a question as to its specific function at this site. We cannot answer this question at this time but can only speculate that, because of its apparent relationship to electrical excitability, the vitamin may be involved in the transport of ions across the cell membrane. It is of interest to note that the concentration of thiamine we find (approximately 10 pmol/mg of protein) corresponds to the number of tetrodotoxin-binding sites on axolemma (28, 31). Some electrophysiological studies with thiamine antimetabolites have suggested an interference with sodium conduction (1–4).

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